Associations of Oxidative Phosphorylation–Related Genes With Deep Intracerebral Hemorrhage in Taiwan

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ABSTRACT

BACKGROUND: Pathway analysis demonstrated associations between deep intracerebral hemorrhage (DICH) and the genetic risk score of complex IV of the oxidative phosphorylation (OXPHOS) pathway in whites. This study investigated the related genetic variations in the DICH population in Taiwan. Candidate variants were selected from the prior report by the following criteria: (1) nuclear genes encoding mitochondria complex IV, (2) genetic effect >1.08, (3) global minor allele frequency >0.01. Six single-nucleotide polymorphisms fitted in the selection criteria, which were mainly involved in Cox assembly, including Cox10, Cox15, and Cox18, and one structural gene, Cox7C. Associations were tested with adjustment of multiple covariables. Permutation testing of 1000 replicates was performed for empirical estimates.

RESULTS: This study enrolled 336 patients and 379 controls. Compared with whites, the Taiwan population has higher minor allele frequency (MAF) of rs4308511, rs767844, and rs221592 and lower MAF of rs8079640. There was no variation of rs16949067 in the Taiwan population. When adjusting for the traditional risk factors, rs221592 G allele was associated with DICH risk in women under additive (odds ratio (OR) = 1.5, 95% confidence interval (CI) = 1.02-2.3, $P = .04$) and recessive models (OR = 2.9, 95% CI = 1.2-6.9, $P = .013$). In an additive fashion, a poor 30-day outcome was associated with rs4308511 T allele (OR = 1.6, 95% CI = 1.1-2.3, $P = .014$) and rs9891372 C allele (OR = 1.7, 95% CI = 1.05-2.8, $P = .024$) in all subjects and in men (rs4308511, OR = 1.8, 95% CI = 1.2-2.7, $P = .008$; rs9891372, OR = 2.1, 95% CI = 1.1-3.8, $P = .02$).

CONCLUSIONS: The results showed ethnic disparities in the complex IV–related genes. COX18-rs221592 G allele was associated with female DICH risks. COX7C-rs4308511 T allele was an independent risk of poor outcome in men.

KEYWORDS: Intracerebral hemorrhage, oxidative phosphorylation, polymorphism, stroke, association study

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Introduction

Primary intracerebral hemorrhage (ICH) is the most devastating stroke subtype with high morbidity and mortality.[1-3] Intracerebral hemorrhage accounted for 8% to 10% of all patients with stroke in Western countries[3,4] but 22% to 35% in Asian populations.[2,5,6] Less than one-third of the ICH patients had a good functional recovery, and the 1-year mortality remained higher than 50%.[1-3,7-9] Most of the ICH (65% to 80%) is caused by hypertension,[10,11] which predominantly occurs at the nonlobar region, including the basal ganglia, thalamus, brain stem, and cerebellum (all are classified as deep ICH [DICH]),[3,9] whereas cerebral amyloid angiopathy–related ICH commonly affects cerebral lobes (lobar ICH).[12,13] The inherited genetic variations of ICH remain largely unexplained by known gene variants.

A recent research pooled several white discovery cohorts (MGH/MIGen) with independent validation cohorts (ISGS/SWISS) and showed associations of oxidative phosphorylation pathway genes (OXPHOS genes) with stroke risks in whites.[14] The genes evaluated in their study included all structural OXPHOS genes and genes contributing to individual respiratory complexes. Using a genetic score based on association significance for single-nucleotide polymorphisms (SNPs) and linkage disequilibrium, they first analyzed the association between risk scores and ischemic stroke, especially small vessel stroke. They also discovered associations between DICH and common genetic variants within complex IV (cytochrome c oxidase, Cox). Although this pathway analysis showed an aggregate association of gene scores with DICH, the study design was not able to identify the specific genetic variants.[14]

Oxidative stress is one of the biochemical consequences that hinder DICH recovery.[15] Several biochemical cascade pathways initiate production of free radicals, which ultimately lead toward neuronal cell death and influence clinical outcome.[16,17] Oxidative stress induces apoptosis through several pathways, such as the mitochondrial pathway and the inflammatory response, triggered by reactive oxygen species (ROS) through the activation of transcription factor nuclear factor κB.[18] Therefore, oxidative phosphorylation–related genes are likely to influence long-term exposures to inflammation and outcomes from DICH. The OXPHOS pathway is the metabolic pathway occurring within mitochondria for internal energy production. Four main protein complexes are involved in the oxidative pathway: NADH-coenzyme Q oxidoreductase

(complex I), succinate-Q oxidoreductase (complex II), electron transfer flavoprotein-Q oxidoreductase, Q-cytochrome c oxidoreductase (complex III), and Cox (complex IV). Cox is the terminal complex in the electron transport chain of oxidation in mitochondria, which controls the proton gradient and establishes a transmembrane difference of proton electrochemical potential for adenosine triphosphate (ATP) synthase. Cox has a complicated structure and contains 13 subunits in mitochondria, 2 heme centers, 2 copper centers, and Fe cofactors. In mammals, the subunits I to III are encoded by mitochondria DNA, whereas subunits IV to VIII are nuclear encoded.

Given the evidence of the association of Cox genetic score with DICH in whites, this study aimed to identify the specific genetic variations of complex IV that were involved in the previously discovered genetic score in terms of the association with the Taiwan DICH.

Methods

Subjects ascertainment

Subjects were recruited from the Department of Neurology, Chang Gung Memorial Hospital (CGMH), Linkou Medical Center. Patients with DICH were diagnosed based on both clinical presentations and computed tomography (CT). Patients with secondary ICH (trauma, brain tumor, vascular anomaly, anticoagulants use, abnormal platelet count, prolonged prothrombin time, and prolonged activated partial thromboplastin time) were excluded. Age-matched participants of the control group were recruited from subjects with no history of neurodegenerative diseases, inflammatory diseases, and stroke. The ethnicity of all of the participants was self-reported as being of Taiwanese Han Chinese ancestry. All the medical records and the outcome were reviewed and assessed by 2 neurologists (Y-C.C. and C-M.C.). All the participants or her or his legally acceptable representative was willing to provide written informed consent to participate. This study was approved by the Institutional Review Board of CGMH.

Anthropometric data and fasting blood samples were collected from all participants. Hypertension was diagnosed when a subject was taking antihypertensive medication to control hypertension or when blood pressure (BP) repeatedly exceeded 140 mm Hg (systolic) and/or 90 mm Hg (diastolic). For DICH cases without prior hypertension, hypertension was recorded when BP measured after the acute phase of DICH (2 weeks within the onset) repeatedly >140 mm Hg (systolic) and/or 90 mm Hg (diastolic). Diabetes mellitus (DM) was defined based on World Health Organization (WHO) criteria. Alcohol use was defined as drinking ⩾210 g per week, as classified as high-risk drinking by National Institute on Alcohol Abuse and Alcoholism. Smoking was defined as former (adults who have smoked at least 100 cigarettes in their lifetime, but say they currently do not smoke) or current smoking (adults who have smoked 100 cigarettes in their lifetime and currently smoke cigarettes daily or nondaily).[19] Hematoma volume at supratentorium, including basal ganglia and thalami, was calculated using the so-called ABC method based on CT of brain.[20] Short-term outcome was assessed using the modified Rankin scale (mRS) on day 30 after DICH events. Subjects who were dead or dependent (mRS > 3) at the 30-day follow-up were considered poor prognosis.[21]

Candidate SNPs selection

From the results of the prior pathway analysis,[14] we have selected candidate genes/SNPs of complex IV by the following criteria: (1) nuclear genes encoding mitochondria complex IV; (2) β-coefficients >0.08 or < −0.08, given the genetic effect of ICH was 1.08 in the prior cohort[14]; and (3) global minor allele frequency (GMAF)>0.01. The β-coefficients indicate the risk of disease when the number of copies of risk allele is increased by one and β-coefficients = 0.08 is relative to odds ratio (OR) = 1.08. There were 6 SNPs selected, mainly involving Cox assembly, including Cox10, Cox15, and Cox18, and one structural gene, Cox7C. The specific information of the 6 selected genetic polymorphisms is listed in Table 1.

Genotyping

Blood samples from patients were collected for SNP genotyping and the genomic DNA was isolated from peripheral leukocytes using DNA Extraction Kit (Stratagene). Genotyping was determined according to a matrix-assisted laser desorption/ionization time-of-flight–based minisequencing genotyping method and the primer sets used for polymerase chain reaction amplification and minisequencing reaction for each SNP region are as listed in Table 2.

Statistics

The Pearson χ^2 test or *t* test was used to compare demographic data between controls and cases; all significance tests were 2-tailed. For SNPs, Hardy-Weinberg equilibrium with significance level set at .05 to control SNP quality. Association analyses were performed first stratified by sex and then combined. Multivariable logistic regression was used to analyze the phenotype-genotype associations of DICH with alleles under additive and recessive genetic models, where appropriate. Covariables included age, sex, hypertension, DM, total cholesterol level, smoking, and alcohol use, which were reported as risks for DICH.[9,15] Factors which may influence the 30-day outcome were evaluated, including age, sex, and the disease severity, such as hematoma size, infratentorial loci, and intraventricular hemorrhage (IVH). Permutation testing of 1000 replicates was performed when the preliminary *P* value was <.05 for empirical estimates as a robust alternative to standard parametric tests. Power calculation was performed by QUANTO version 1.0. In the present case-control study, at the 5% significance level, we had power greater than 0.8 to identify

Table 1. Information of the selected genetic polymorphisms.

GENE	SNP	B-COEFFICIENT	LOCI	GMAF	FUNCTION
COX7C	rs4308511	1.235	chr5, 85868303	T: 0.0723	Unknown
COX10	rs9891372	−0.0815	chr17, 14045853	C: 0.1575	Intron
COX10-AS1	rs16949067	0.1989	chr17, 14037909	G: 0.0554	Intron
COX10-AS1	rs8079640	−0.0875	chr17, 14037307	A: 0.1658	Intron
COX15	rs767844	−0.2565	chr10, 101500471	G: 0.1387	Intron
COX18	rs221592	−0.0809	chr4, 73710342	G: 0.1845	Unknown

From the results of GWAS and pathway analysis, we have selected candidate genes/single-nucleotide polymorphisms (SNPs) of complex IV pathway. Global minor allele frequency (GMAF) in each SNP is shown. The MAF in the 1000 Genome phase 1 population in the sample population of 629 people (or 1258 chromosomes) is demonstrated.

Table 2. Primers used for amplification and minisequencing reaction with matrix-assisted laser desorption/ionization time-of-flight–based minisequencing genotyping method.

GENE	SNP	PRIMERS
COX7C	rs4308511	F: CTGACCTTCGTTCCTTCAGC R: TGGGGTTCTTTACTACCGATACA Product size: 248 bp MSP: GCTAGAGTGGAAGACAT
COX10	rs9891372	F: TTTGCCTGCTTTCTGAGTTAAA R: TGGGAAGAAATGTTTGCTTTT Product size: 216 bp MSP: GGTATTTGGCAGAAGAGT
COX10-AS1	rs16949067	F: TTGGGAGATGAAAAGCATCC R: CCACACATATTCTTTGGGAACA Product size: 169 bp MSP: AGGGACAGCAAGCACAT
COX10-AS1	rs8079640	F: TGTTTTCATATGGAAGTCTTTTCTTTT R: AATTTGAGGGCATCATGGAG Product size: 244 bp MSP: TAAGAGGAACCCTGGTATTC
COX15	rs767844	F: TCAGCTTGCAGCTTGTTCTC R: GCTCAAGGACAAAACAATAGCA Product size: 165 bp MSP: CTCCTTTAGAGACTTAAGCT
COX18	rs221592	F: CCTAGCCTTGGACAAGCATT R: GATGGTTATCTTGCGAAGCA Product size: 175 bp MSP: ATTTTTTTCTGGCCTTGTTA

Abbreviations: F, forward primer; MSP, minisequencing primer; R, reverse primer; SNP, single-nucleotide polymorphism.

an association under an additive genetic model when the per-allele genetic effect was greater than 1.5 when the MAF >0.1. Analyses were performed using SAS software version 9.1.3 (SAS Institute, Cary, NC, USA).

Results

The demographic data for the enrolled population are presented in Table 3. A total of 336 DICH patients and 379 controls were included in this study. The proportions of hypertension, smoking, and alcohol consumption were significantly higher in patients than in controls. Total cholesterol was borderline higher in DICH patients than in controls ($P = .04$). There was no sex difference in the 30-day poor outcome (mRS > 3) and 1-year recurrent stroke in DICH patients. The 30-day poor outcome was affected by age (OR = 1.04, 95% confidence interval (CI) = 1.2-1.06, $P = .0003$) and hemorrhage size (OR = 1.06, 95% CI = 1.04-1.09, $P < .0001$) but was not affected by sex, infratentorial loci, and intraventricular extension. Because the male sex carries a risk for DICH and male patients tended to have a larger hemorrhage size, analyses were further performed with sex stratification when we examined the genetic effect on DICH.

Frequency and association of each genotype in DICH and control subjects are shown in Table 4. There was no difference

Table 3. Demographics of the study population.

	MALES (N = 420)			FEMALES (N = 295)			BETWEEN SEX
	DICH	CONTROLS	*P* VALUE	DICH	CONTROLS	*P* VALUE	*P* VALUE
	N = 234	N = 186		N = 102	N = 193		
Age, y	58.0 ± 12.8	60.0 ± 11.3	.11	64.2 ± 12.3	62.0 ± 8.5	.09	.04
Hypertension, %	86.8	49.5	<.0001	92.2	43.5	<.0001	.16
Diabetes mellitus, %	15.0	18.5	.35	21.6	15.3	.18	.14
Alcohol use, %	39.3	21.0	<.0001	2.9	1.0	.23	<.0001
Smoke, %	57.7	30.1	<.0001	4.9	2.1	.18	<.0001
Body mass index, kg/m^2	25.4 ± 4.2	25.5 ± 3.3	.90	24.8 ± 4.2	25.2 ± 3.6	.35	.52
Total cholesterol, mg/dL	173.6 ± 44.2	163.5 ± 60.7	.04	176.0 ± 46.4	168.9.0 ± 65.2	.28	.38
Infratentorial %	26.9			20.6			.22
IVH, %	4.7			5.9			.65
Hemorrhage size, mL	17.0 ± 18.6			12.3 ± 11.5			.02
30-day mRS > 3, %	41.9			43.1			.83
1-y recurrent stroke, %	9.8			8.8			.77

Abbreviation: DICH, deep intracerebral hemorrhage; IVH, intraventricular hemorrhage.
Data are expressed as percentage or mean ± SD. Hemorrhage size was the calculated in patients with supratentorial hemorrhage.
Comparisons between controls and DICH group were analyzed using Pearson χ^2 test or *t* test where appropriate.
To convert mg/dL to mmol/L, multiply cholesterol values by 0.02586.

Table 4. Frequencies and associations of the genotypes in patients with deep intracerebral hemorrhage (DICH) and controls.

GENOTYPES	MALES, N = 420			FEMALES, N = 295			ALL
	DICH, % N = 234	CONTROLS, % N = 186	*P* VALUE[A,B]	DICH, % N = 102	CONTROLS, % N = 193	*P* VALUE [A,B]	*P* VALUE [A,B]
rs4308511 CC/CT/TT	59.8/30.3/9.8	54.1/38.9/7	.15, .73	53.9/42.2/3.9	58.6/35.6/5.8	.48, .78	.52, .61
rs9891372 TT/TC/CC	77.7/21/1.3	70.7/28.2/1.1	.24, .22	76.2/20.8/3	77.1/21.3/1.6	.72, .96	.46, .35
rs16949067 AA	100	100	–	100	100	–	–
rs8079640 GG/GA/AA	76.8/21.9/1.3	69.2/29.7/1.1	.19, .56	76.2/20.8/3	77.6/21.4/1	.48, .26	.36, .23
rs767844 AA/AG/GG	46.6/45.3/8.1	47.6/40/12.4	.27, .77	48.1/43.1/8.8	48.4/43.2/8.3	.99, .75	.56, .75
rs221592 TT/TG/GG	46.2/40.6/13.2	51.9/37.3/10.8	.47, .66	47.5/34.7/17.8	54.2/37.9/7.9	.04, .04[c]	.06, .13

[a]*P* value was examined by χ^2 test with allele frequency.
[b]*P* value was derived by logistic regression under additive genetic model, adjusting for age, sex, hypertension, DM, alcohol drinking, smoking, and total cholesterol level.
[c]Odds ratio = 1.5, 95% confidence interval 1.02 to 2.3.

between cases and controls regarding the genotype distribution of the candidate SNPs in the overall cohort. When stratified by sex, rs221592 was associated with DICH risk in women under additive (OR = 1.5, 95% CI = 1.02-2.3, *P* = .04) and recessive genetic model (OR = 2.9, 95% CI = 1.2-6.9, *P* = .013). There was no association between the candidate genes and DICH risks in men (Table 4). The MAF of rs4308511, rs9891372, rs8079640, rs767844, and rs221592 is 0.25, 0.13, 0.018, 0.31, and 0.31, respectively. Except for rs221592, in which the MAF distribution was significantly different between DICH group and controls (*P* = .02, data not shown in Table 4), there was no significance in MAF distribution between cases and controls.

For the 30-day outcome, we found that poor 30-day outcome (mRS > 3) was associated with rs4308511 (OR = 1.6, 95% CI = 1.1-2.3, *P* = .014) and rs9891372 (OR = 1.7, 95% CI = 1.05-2.8, *P* = .024) (Table 5). The significant associations between the

Table 5. Prediction of poor outcome (30-day mRS > 3) of the patients with deep intracerebral hemorrhage.

	MALES, N = 234			FEMALES, N = 102			ALL
	MRS > 3, % N = 98	MRS ⩽ 3, % N = 136	*P* VALUE[A,B]	MRS > 3, % N = 44	MRS ⩽ 3, % N = 58	*P* VALUE[A,B]	*P* VALUE[A,B]
rs4308511 CC/CT/TT	49/36.7/14.3	67.7/25.7/6.6	.008†[a], .003†[b]	52.3/43.2/4.5	55.2/41.4/3.5	.49, .13	.014§[a], .002§[b]
rs9891372 TT/TC/CC	70.4/27.6/2	83/16.3/0.7	.02‡, .16	76.2/20.8/3	76.7/16.3/7	.70, .93	.024[a], .20
rs16949067 AA	100	100	—	100	100	—	—
rs8079640 GG/GA/AA	70.1/27.8/2.1	81.6/17.7/0.7	.79, .93	76.7/16.3/7	75.9/24.1/0	.98, .96	.14, .19
rs767844 AA/AG/GG	53.1/36.7/10.2	41.9/51.5/6.6	.40, .70	54.6/40.9/4.6	43.1/44.8/12.1	.20, .08	.15, .14
rs221592 TT/TG/GG	45.9/37.8/16.3	46.3/42.7/11	.59, .51	53.5/34.9/11.6	43.1/34.5/22.4	.36, .19	.86, .87

[a]*P* value was derived by logistic regression under additive genetic model, adjusting for age, sex, hypertension, DM, alcohol drinking, smoking, and total cholesterol level.
[b]*P* value was derived by logistic regression under additive genetic model, adjusting for age, sex, hypertension, DM, alcohol drinking, smoking, total cholesterol level, and hemorrhage size.
†[a]Odds ratio (OR) = 1.8, 95% confidence interval (CI) = 1.2 to 2.7; †[b] OR = 2.01, 95% CI = 1.3 to 3.2.
‡OR = 2.1, 95% CI = 1.1 to 3.8.
§[a]OR = 1.6, 95% CI = 1.1 to 2.3; §[b] OR = 1.9, 95% CI = 1.3 to 2.9.
OR = 1.7, 95% CI = 1.05 to 2.8.

SNPs and the outcome were mainly in the male group (rs4308511: OR = 1.8, 95% CI = 1.2-2.7, *P* = .008; rs9891372: OR = 2.1, 95% CI = 1.1-3.8, *P* = .02). The significance of the association between rs4308511 and outcome remained when further adjusted for hemorrhage size (OR = 2.01, 95% CI = 1.3-3.2, *P* = .003), suggesting an independent role of rs4308511 on DICH outcome in the male patients. However, the association between rs9891372 and outcome was not significant when further adjusted for hemorrhage size. There was no association between the candidate SNPs and the 30-day mortality and 1-year recurrent DICH rate.

Discussion

This is the first study that investigated associations of complex IV–related genes with DICH risks and outcome in the Taiwan population. Carrying rs221592 G allele (*COX18*) was a risk for DICH in women. This study also demonstrated that carrying rs4308511 T allele (*COX7C*) was an independent risk for a poor 30-day outcome after DICH, especially in men. There was an ethnic difference in the distribution of OXPHOS pathway genes. Specifically, all of the examined subjects have rs16949067 AA genotypes. In addition, there was higher MAF of rs4308511, rs767844, and rs221592 and lower MAF of rs8079640 in the Taiwan population (0.25, 0.31, 0.31, and 0.018, respectively) than in whites (GMAF 0.07, 0.14, 0.18, and 0.17, respectively), suggesting ethnic disparities in the Cox-related genes.

In the pooled white cohorts, Anderson et al[14] showed associations of complex IV genes with DICH risks in whites. Although they discovered associations between DICH and common genetic variants within complex IV, their study design highlights an aggregate association and therefore cannot identify specific genetic variants.[22] The study herein supports the prior finding of associations between complex IV–related genes and DICH risks and highlights the variants of rs221592 (*COX18*), rs4308511 (*COX7C*), and rs9891372 (*COX10*) in the Taiwan DICH risks.

The involvement of the complex IV genes in DICH may be contributing to mitochondria dysfunctions including ATP depletion and generation of ROS, which would affect cell survival. Oxidative stress has been shown to result in increased expression of adhesion molecules and blood-brain barrier permeability. When a small artery ruptures and the hematoma bleeds into the surrounding brain, various cellular and molecular components determining neuronal survival were activated,[23] and the inflammation response was triggered by ROS through the activation of transcription factor nuclear factor κB.[24] We have shown that increased leukocyte 8-hydroxy-2-deoxyguanosine (8-OHdG) as well as decreased erythrocyte glutathione peroxidase and plasma vitamin E during acute DICH, in which 8-OHdG was associated with ICH and the 30-day outcome independently from the other oxidative markers and the traditional factors.[15] Mitochondria dysfunction may lead to poor compensation during the oxidative stress after DICH and thus lead to a poor outcome.

This study has several strengths. First, this study included a homogeneous disease entity in a same ethnic background between the case and the control groups, which may limit the confounding effect from multiple phenotypes and ethnicities. Second, this is the first study analyzing associations of individual complex IV genes with the DICH susceptibility. This allows us to identify which SNPs may be used as tag variants. However, this study has several limitations. First, the number of the DICH patients is relatively small. Furthermore, a replicated study is needed to confirm the results herein, especially for the female population to avoid a false-negative result. In

addition, the SNPs may not pose a specific risk factor for DICH, given that these genes have been shown to be a risk for other diseases.[25] Second, we did not record data of medication use, such as antiplatelet agent and statin, which may influence the clinical outcome. Third, there is also a limitation that the ethnicity was self-reported, in which the reliability was uncertain. Furthermore, because the effect sizes were modest, the result needs to be replicated before these SNPs can be viewed as independent risk factors for DICH and its outcome.

The results showed ethnic disparities in the complex IV–related genes. There was significant association of rs221592 G allele (*COX18*) with female DICH risk in additive and recessive fashions. Carrying rs4308511 T allele (*COX7C*) and rs9891372 C allele (*COX10*) were both risks of poor 30-day outcome after DICH in men.

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Author Contributions

YCC and CMC recruited participants. YCC and YSL designed the study, selected candidate variations, and analyzed and interpreted the data. YCC, CMC, and KHC make contributions to conception and design of this study. YCC made contributions to genotyping and acquisition of data.

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